MAGNETIC RESONANCE IN CHEMISTRY
Magn. Reson. Chem. **36**, S79–S84 (1998)

Assignment of mono- and polyunsaturated fatty acids in lipids of tissues and body fluids†

Wieland Willker and Dieter Leibfritz*

Institut für Organische Chemie, Universität NW2, 28334 Bremen, Germany

Received 2 December 1997; revised 23 January 1998; accepted 23 January 1998

ABSTRACT: Semi-selective 800 MHz ^{1}H, ^{13}C HSQC NMR spectra provide ultra-high resolution of the double bond region of lipid spectra and the possibility of assigning signals to individual unsaturated fatty acid side-chains such as 18:1, 18:2 or 20:4 even in complex mixtures such as biofluids. Additional HSQC-TOCSY spectra correlate the direct double bond signals with the adjacent allylic high-field proton signals of mono- and polyunsaturated fatty acids. These signals show characteristic chemical shifts to differentiate unsaturated fatty acids such as 18:1, 18:2 and 20:4 (or others). By this means, the amount of each type of unsaturated fatty acid can be quantified. Typical lipid spectra of body fluids (blood plasma and cerebrospinal fluid) and pig brain (grey and white matter) are presented and discussed with respect to the use of this technique for diagnostic purposes. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; ^{13}C NMR; fatty acids; ultra-high field; lipids; body fluids

INTRODUCTION

Unsaturated fatty acids are essential for modulating the mechanical properties and function of cell membranes. Several human diseases show abnormal patterns of unsaturated fatty acids attributable to abnormal metabolism, especially diminished capabilities for desaturation or chain elongation.[1] The analysis of alterations in the lipid composition is also a major factor in understanding neoplasticity and the treatment of cancer. Therefore, analytical tools are required to distinguish and assign individual unsaturated fatty acids in body fluids. In principle, NMR is very well suited for this purpose, because different pure unsaturated fatty acids can be differentiated by their spectra. However, a huge signal overlap obscures the 1D ^{1}H spectrum of mixtures. The 1D ^{13}C spectrum, on the other hand, provides excellent resolution and separation of many double bond signals, but suffers from low sensitivity. Several unsaturated fatty acids have been assigned in the 1D ^{13}C spectra of tissue and blood plasma.[2–10] Other groups used 1D ^{1}H spectra for the calculation of an unsaturation index.[7,10–12] Some allylic signal assignments have been made by Casu *et al.*[13] in 1D ^{1}H spectra of rat liver. In this work, we used semi-selective HSQC and HSQC-TOCSY experiments[14–16] to assign the double bond proton and carbon signals and signals of neighbouring protons whose chemical shifts are affected by the double bonds. Several of these allylic signals can be identified in the 1D ^{1}H spectra because of their characteristic ^{1}H chemical shifts. By this means, 18:1($n-9$), 18:2($n-6$), 20:4($n-6$) and 22:6($n-3$) can be identified and quantified.

* Correspondence to: D. Leibfritz, Institut für Organische Chemie, Universität NW2, 28334 Bremen, Germany.
† Dedicated to Professor John D. Roberts on the occasion of his 80th birthday.

EXPERIMENTAL

Spectra

All experiments were performed on Bruker DRX 600 and 800 MHz spectrometers at 300 K using a 5 mm H, C, N inverse triple resonance probe with actively shielded field gradient coils. Gradients were shaped by a waveform generator and amplified by a Bruker Acustar amplifier. Sinusoidal z-gradients of 1 ms duration and a recovery time of 100 ms were used for the echo/antiecho gradient selection. Fine tuning of the gradient amplitude ratios (40:10.08) resulted in optimum signal intensities. Low-power adiabatic composite pulse decoupling with WURST[17] was used for ^{13}C decoupling. A sensitivity-improved HSQC[18,19] in the version described in Ref. 15 with a relaxation delay of 1 s was used. The semi-selective HSQC experiments were acquired with 1K increments in F_1 and 24 scans per increment in 13 h to obtain a resolution of 0.7 Hz in F_1. An acquisition time of 285 ms was used to acquire 1024 data points for a spectral width of 3 ppm in the proton dimension. Folded signals were suppressed using digital quadrature detection (DQD) (Bruker). Details of the semi-selective HSQC can be found elsewhere.[14,15] The semi-selective HSQC-TOCSY experiment was sampled with 1K increments in F_1 and 48 scans per increment in 23 h to obtain a resolution of 1.1 Hz in F_1. A mixing time of 40 ms was used. The 1D ^{13}C spectrum was recorded with a repetition time of 3 s and 78K scans in

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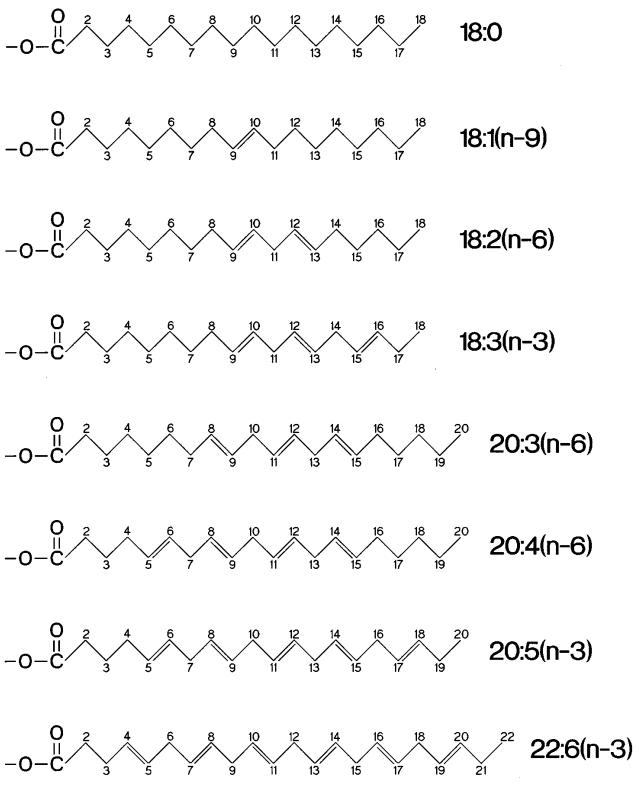

Scheme 1.

3 days 20 h. All spectra are pure absorption mode spectra and were processed with a $\pi/2$ shifted squared sine-bell in F_2 (1H) and a $\pi/4$ shifted squared sine-bell in F_1 (^{13}C).

Samples

The lipids were extracted using a modified Folch procedure.[20] The lyophilized body fluids [5 ml of blood plasma and 70 ml of cerebrospinal fluid (CSF) combined from four patients] were extracted three times with chloroform–methanol (2:1), the first time with 4.5 ml then twice with 1.5 ml. The combined extracts were washed once with 1/4 volume of water and once with 1/4 volume of water–methanol (1:1). The(upper) water phase was removed in both cases. The solvents were evaporated in a stream of nitrogen and the lipids were dissolved in 0.6 ml of deuterated chloroform–methanol (2:1). Samples of pig brain tissue were homogenized and centrifuged. The pellet was extracted in the same way as described above.

Nomenclature

Fatty acid chains (F) are abbreviated using the conventional notation, e.g. 18:2($n-6$), which indicates a fatty acid with an 18-carbon chain, with two double bonds and with the first double bond six atoms away from the CH_3 group (e.g. carbon number 13). The subsequent double bond (Δ) is separated from the first by one CH_2 group (see Scheme 1). For more details, see Ref. 21.

RESULTS AND DISCUSSION

Figure 1 shows sections of the 600 MHz 1D ^{1}H spectrum of blood plasma lipids, illustrating (a) the double bond region and (b) the signal region of allylic Δ–CH_2–Δ and $\Delta-1$ protons and the F_α and F_β protons adjacent to the carboxyl group. The double bond protons of the unsaturated fatty acids are all superimposed at 5.3–5.4 ppm. Unsaturated fatty acids 18:1($n-9$), 18:2($n-6$) and 20:4($n-6$) constitute the predominant part of blood plasma unsaturated fatty acids.[22] Other minor components are 18:3($n-3$), 20:3($n-6$), 20:5($n-3$) and 22:6($n-3$).

Figure 2 shows a semi-selective 800 MHz HSQC spectrum of this double bond region with ultra-high resolution of 0.7 Hz per point in F_1(^{13}C). Although all the signals show very similar chemical shifts in the proton dimension, they are very well separated in the carbon dimension. The resolution is almost the same as in the 1D ^{13}C spectrum shown on the left. By comparison with reference compounds, the signals can be assigned to the different unsaturated fatty acid side-chains which show fingerprint-like patterns as indicated

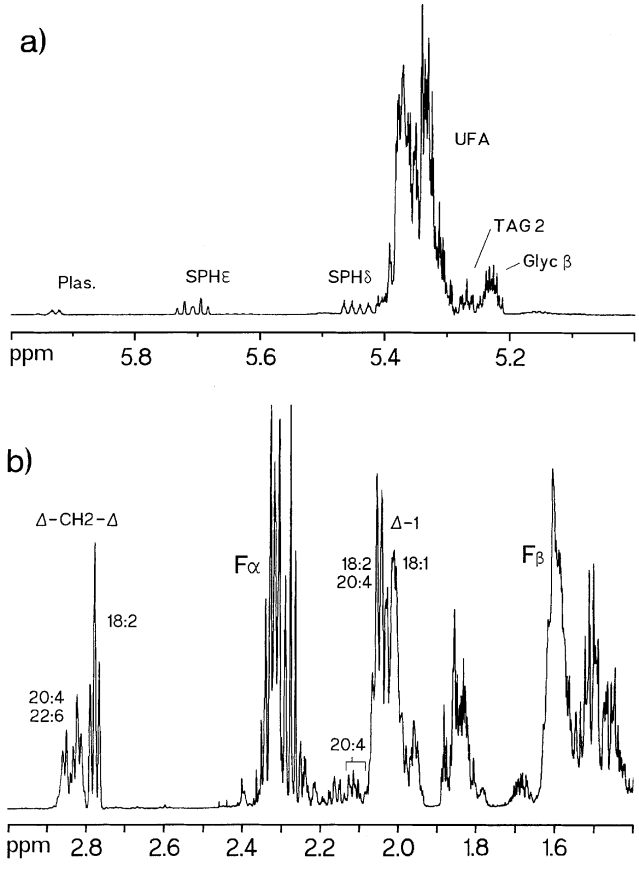

Figure 1. 600 MHz ^{1}H spectrum of blood plasma lipids. (a) Double bond section. Plas = plasmalogen α-signal; SPH = sphingomyelin; UFA = unsaturated fatty acids; TAG = triacylglycerids; Glyc = glycerol headgroup of phospholipid. (b) High-field region of protons adjacent to the double bonds. F_α, F_β = protons in the fatty acid chain.

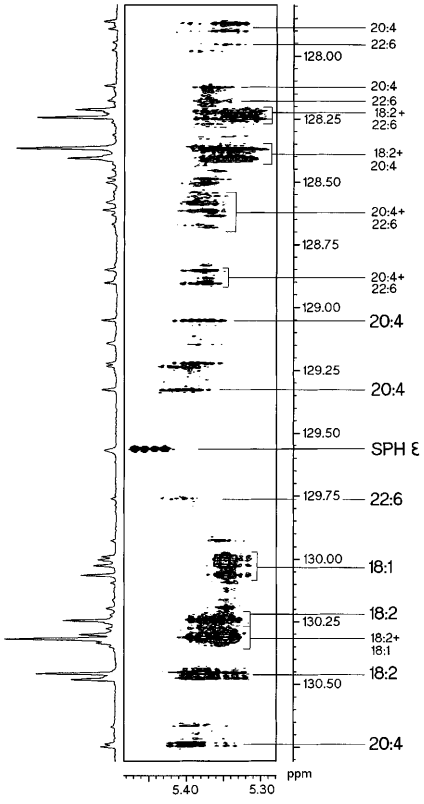

Figure 2. Semi-selective 800 MHz HSQC spectrum of the double bond region of blood plasma lipids. The 1D ^{13}C spectrum is shown on the left. The various signal regions have been assigned to individual fatty acid side-chains with the aid of reference compounds.

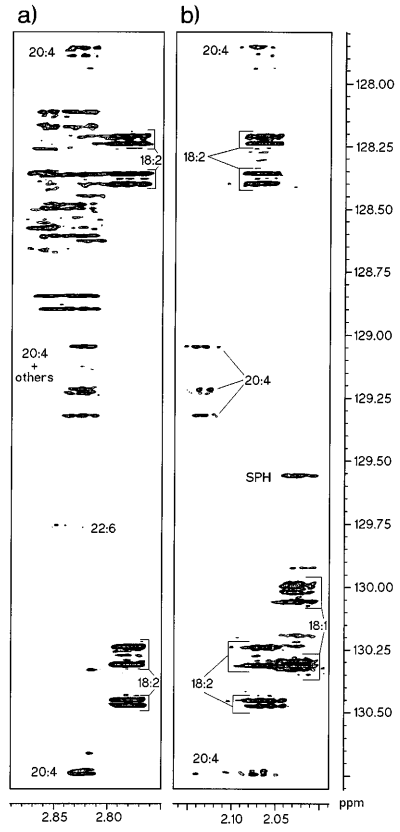

Figure 3. Semi-selective 800 MHz HSQC-TOCSY spectrum of the double bond region of blood plasma lipids. (a) Correlations to the allylic Δ–CH_2–Δ protons (*ca.* 2.8 ppm); (b) $\Delta - 1$ CH_2 protons (*ca.* 2.05 ppm). In the Δ–CH_2–Δ region the 18:2 signals are well separated from the higher polyunsaturated fatty acids, whereas in the $\Delta - 1$ region the 18:1 signals are separated from all other signals. The 20:4 fatty acid shows additional well separated signals at 2.13 ppm.

for 18:1, 18:2 and 20:4. Approximately 60 signals can be distinguished in this region. Small signal splittings in the carbon dimension of a particular unsaturated fatty acid indicate the presence of different glycerophospholipid isoforms such as varying fatty acids at the glycerol γ-position (e.g. 16:0 or 18:0), or phospholipids with different polar headgroups. The ^{13}C chemical shifts of the measured reference compounds are given in Table 1.

The semi-selective HSQC-TOCSY spectrum of the same carbon region is shown in Fig. 3. Fig. 3(a) depicts the correlations of the olefinic carbons and the allylic Δ–CH_2–Δ proton signals (*ca.* 2.8 ppm). The 18:2 signals are well separated from the higher unsaturated fatty acids. Figure 3(b) shows the correlations of the olefinic carbons and $\Delta - 1$ proton signals (*ca.* 2.1 ppm). Here the allylic proton signal of the 18:1 fatty acid is well separated from the corresponding proton signals of the other unsaturated fatty acids, except sphingomyelin $\Delta - 1$, which has the same proton shift. Compounds 18:2 and 20:4 can be distinguished because 20:4 shows two isolated signals at 2.06 (H16) and 2.13 ppm (H4).

Figure 4 shows the corresponding direct $^1H/^{13}C$ HSQC correlation signals of Δ–CH_2–Δ and $\Delta - 1$ CH_2. Using the HSQC-TOCSY information, individual contributions to these normally overlapping 'signal bulks' can be assigned to 18:1, 18:2 and 20:4. Table 2 gives the $^1H/^{13}C$ assignment of all high-field signals influenced by double bonds.

As some of these signals are also well separated in the 1D proton spectrum [Fig. 1(b)], we can now estimate the amount of the unsaturated fatty acids by integration using the assignment obtained from the HSQC-TOCSY spectrum. Figure 5 compares the Δ–CH_2–Δ region of (a) blood plasma, (b) CSF and pig brain, (c) white and (d) grey matter lipids. The integrals of these signals together with the 18:1 $\Delta - 1$ signal integral obtained at 2.02 ppm are shown in Fig. 6. The integrals are given in relative amounts *vs.* the F_Δ signal 'hump' at 5.2–5.3 ppm (100%). The amount of 18:2 fatty acids (pseudo-triplet

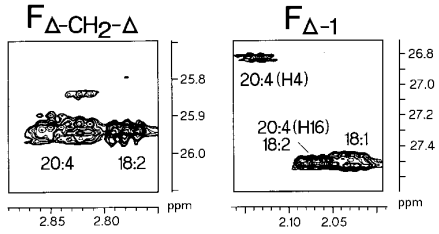

Figure 4. Semi-selective 800 MHz HSQC spectrum with the Δ–CH_2–Δ and $\Delta - 1$ proton signals of unsaturated fatty acid chains in blood plasma. The same proton regions as in Fig. 3 are used to allow a direct comparison.

Table 1. Carbon chemical shifts of phosphatidylcholine with different unsaturated FA at the Glyc β position measured in $CDCl_3$–MeOH (2:1)[a]

Fatty acid	δ (ppm)	Fatty acid	δ (ppm)
18:1 ($n-9$)	130.29 (C10)	20:5 ($n-3$)	132.92 (C18)
	129.96 (C9)		129.31 (C6)
18:2 ($n-6$)	130.47 (C13)		129.11 (C5)
	130.24 (C9)		128.84
	128.41 (C10)		128.53
	128.22 (C12)		128.53
18:3 ($n-3$)	132.23 (C16)		128.418
	130.54 (C9)		128.404
	128.602		128.19
	128.594		127.35 (C17)
	128.13 (C10)	22:6 ($n-3$)	132.26 (C20)
	127.50 (C15)		129.71 (C5)
20:3 ($n-6$)	130.66 (C15)		128.82
	130.41		128.63
	128.62		128.55
	128.52		128.55
	128.22		128.328
	128.01 (C14)		128.321
20:4 ($n-6$)	130.71 (C15)		128.23
	129.29 (C6)		128.14
	129.02 (C5)		127.94 (C4)
	128.87		127.30 (C19)
	128.58		
	128.34		
	128.09		
	127.83 (C14)		

[a] Shifts referenced to TMS (0 ppm), equivalent to MeOD = 48.93 ppm.

at 2.78 ppm) varies considerably within this series. While brain tissue extracts from pig contain only minor amounts of fatty acid 18:2, the human blood and CSF show distinctly higher amounts. 18:2 is the predominant polyunsaturated fatty acid in human blood. Interesting differences are also present in the different brain matter. White matter contains less polyunsaturated fatty acids than grey matter, which is consistent with the contribution of oligodendrocytes to the myelin sheaths.

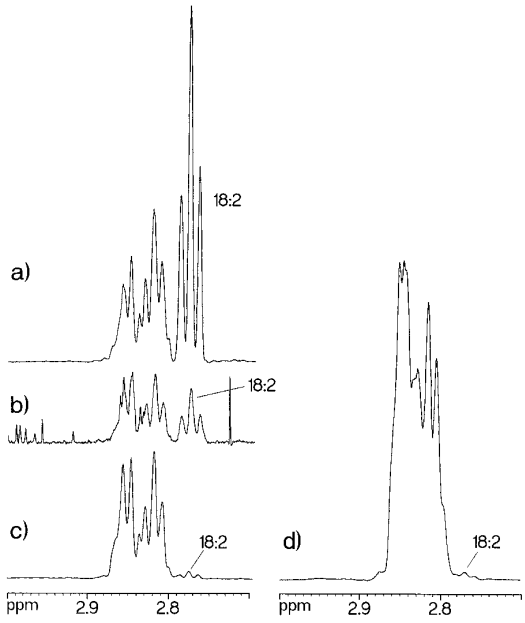

Figure 5. Comparison of the Δ–CH_2–Δ section in 1D 600 MHz ^1H spectra of different lipid extracts. (a) Blood plasma; (b) CSF; (c) pig brain (white matter); (d) pig brain (grey matter).

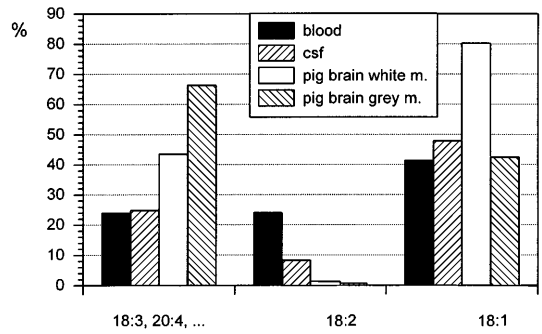

Figure 6. Signal integrals of the Δ–CH_2–Δ regions and the 18:1 $\Delta-1$ CH_2 group from the spectra shown in Fig. 5. The left part of the Δ–CH_2–Δ signal represents all polyunsaturated fatty acids with three and more double bonds. The right part (2.78 ppm) represents 18:2 only. The total F_Δ signal area was used as an internal reference (100%) for unsaturated fatty acids. The 18:1 signal was corrected by subtraction of the amount of superimposed cholesterol C7 and C12 signals and the sphingomyelin $\Delta-1$ signal.

Table 2. High-field chemical shifts of different unsaturated fatty acids[a]

Fatty acid	Signal	$^1H/^{13}C$ in ppm
18:1 ($n-9$)	$\Delta-1$ (H8,11)	2.02/27.45
	F_α (γ-chain)	2.31/34.37
	F_α (β-chain)	2.33/34.51
	F_β	1.61/25.18
	F_ω	0.88/14.17
18:2 ($n-6$)	Δ–CH_2–Δ	2.78/25.93
	$\Delta-1$ (H8,14)	2.06/27.50
	F_α (γ-chain)	2.32/34.41
	F_α (β-chain)	2.34/34.54
	F_β	1.61/25.23 + 25.20
	$F_{\omega-3}$ ($\Delta-2$) H15	1.30/31.85
	F_ω (γ-chain)	0.885/14.17
	F_ω (β-chain)	0.897/14.20
18:3 ($n-3$)	Δ–CH_2–Δ	2.82/25.96 + 25.86
	$\Delta-1$ (H8)	2.069/27.54
	F_α	2.32/34.44
	F_β	1.63/25.29
	$F_{\omega-1}$ ($\Delta-1$)	2.088/20.88
	F_ω ($\Delta-2$)	0.98/14.42
20:3 ($n-6$)	$\Delta-1$ (H7,16)	2.068/27.559 + 2.074/27.491
	Δ–CH_2–Δ	2.82/25.97
	F_α	2.32/34.41
	F_β	1.63/25.27
	F_ω	0.901/14.21
20:4 ($n-6$)	Δ–CH_2–Δ	2.85 + 2.82($\times$2)
		25.90 + 25.884 + 25.875
	$\Delta-1$ (H16)	2.06/27.48
	$\Delta-1$ (H4)	2.13/26.77
	F_α (γ-chain)	2.31/34.35
	F_α (β-chain)	2.36/33.93
	F_β (γ-chain)	1.60/25.15
	F_β (β-chain)	1.70/25.08
	F_ω (γ-chain)	0.882/14.14
	F_ω (β-chain)	0.894/14.16
20:5 ($n-3$)	Δ–CH_2–Δ	2.854/25.911 + 25.907
		2.822/25.897
		2.820/25.811
	F_α	2.32/33.82
	F_β	1.70/25.12
	$\Delta-1$ (H4)	2.134/26.87
	$F_\omega-1(\Delta-1)$	2.083/20.82
	F_ω	0.98/14.37
22:6 ($n-3$)	Δ–CH_2–Δ	2.86 + 2.82
		25.89 + 25.88($\times$2) + 25.84 + 25.78
	F_β ($\Delta-1$)	2.40/22.85
	$F_{\omega-1}$ ($\Delta-1$)	2.08/20.79
	F_ω ($\Delta-2$)	0.978/14.36
	F_α (γ-chain)	2.31/34.35
	F_α (β-chain)	2.41/34.33
	F_β (γ-chain)	1.60/25.13
	F_ω	0.881/14.18

[a] 18:1, 18:2, 20:4 and 22:6 were measured as phosphatidylcholine, 18:3 and 20:3 as the methyl ester and 20:5 as the free acid in $CDCl_3$–MeOH (2:1). Shifts referenced to TMS (0 ppm), equivalent to MeOD = 3.349/48.93 ppm.

Further details of the unsaturated fatty acid composition can be obtained from the semi-selective 2D spectra. Integrals of signals characteristic for higher unsaturated fatty acids are depicted in Fig. 7. The percentages are given in relative amounts with respect to the F_ω signal at 0.88 (^1H)/14.2 (^{13}C) ppm. The two signals at 2.08/20.8 ppm ($F_{\omega-1,\Delta-1}$) and at 0.98/14.4 ppm [$F_{\omega(n-3)}$] represent all ($n-3$) polyunsaturated fatty acids, mainly

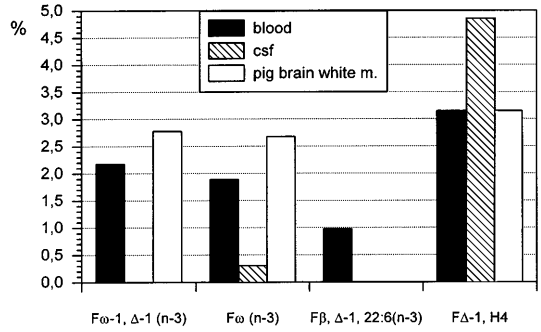

Figure 7. Signal integrals obtained from 2D spectra by volume integration of the relevant signals. The first two signals [$F_{\omega-1,\Delta-1}$ and $F_{\omega(n-3)}$] represent all $(n-3)$ polyunsaturated fatty acids (18:3, 20:5 and 22:6), the third signal represents 22:6$(n-3)$ only and the fourth signal represents 20:4$(n-6)$ plus 20:5$(n-3)$. The unaffected F_ω signal was used as an internal reference (100%) for quantification.

18:3; 20:5 and 22:6. Both integrals correspond to each other very well, e.g. 2–2.5% of the total amount of fatty acids. The integral of $F_{\beta(\Delta-1)}$ at 2.40/22.85 ppm specifically represents 22:6$(n-3)$ and was found in blood only. The relative amount of this polyunsaturated fatty acid is *ca.* 1% in blood plasma. Subtraction from the first two integrals leaves another *ca.* 1% for the 18:3$(n-3)$ and 20:5$(n-3)$ polyunsaturated fatty acids. Finally, the signal $\Delta-1$(H4) at 2.13/26.8 ppm is typical for 20:4$(n-6)$ and 20:5$(n-3)$. Subtracting 1% for 20:5$(n-3)$ gives an amount of 3–4% for 20:4$(n-6)$.

CONCLUSIONS

Semi-selective HSQC experiments provide sufficient spectral resolution to acquire ultra-high resolved lipid spectra with improved signal-to-noise ratio compared with 1D ^{13}C spectra. The spectra offer the possibility of distinguishing and quantifying various mono- and polyunsaturated fatty acids individually in one sample without prior separation. This is a prerequisite for a fast screening method for biofluids for medical diagnosis. The quantification was performed on selected samples to prove its technical feasibility. Future studies will have to corroborate general conclusions on the lipid composition of samples by comparing normal and pathological states as well as samples from different sources.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft for a 600 MHz NMR spectrometer and access to the 800 MHz spectrometer in Frankfurt a. M., Germany and the University of Tübingen (Fortune programme) for a fellowship (W.W.).

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